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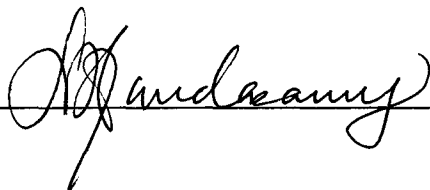
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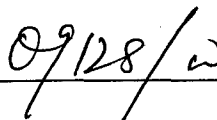
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13. ABSTRACT (Maximum 200 words) My lab is developing techniques for imaging living human breast epithelial cells in 3-D cultures to evaluate their responses to the application of exogenous factors. Unlike most model systems, which utilize cells growing in monolayers on plastic substrates, our system more closely mimics the growth of cells in the body. Recent data from other laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same manner as do cells growing in three-dimensional cultures. There is a strong need, therefore, for techniques such as ours that enable examination of living cells growing in 3-D. We can now track fluorescently labeled proteins in living cells and, thereby, evaluate "normal," premalignant and tumor cells. For example, we can visualize β -catenin-GFP in live normal and tumor breast cells. Using such approaches, we can detect rapid responses to the effects of exogenous factors, including therapeutic agents. Other preliminary studies of proteins in the Wnt signaling pathway (known to be involved in a number of cancers) in fixed human mammary epithelial cells show that one of the key proteins is absent in the tumor cells. Further studies using our technique are required to determine the significance of this aberration.				
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FOREWORD

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Carolyn Larell Aug 12, 1999
PI - Signature Date

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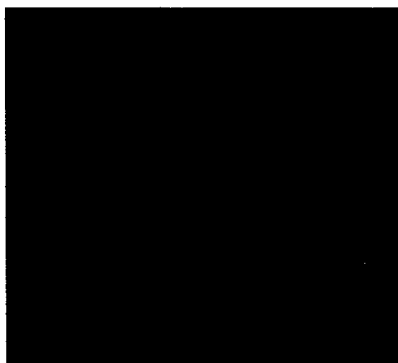
(5) INTRODUCTION

The focus of this proposal is to develop a technique for imaging living human breast epithelial cells in 3-D cultures and to evaluate their responses to the application of exogenous factors. Most model systems used to study breast cancer utilize cells growing in monolayers on plastic substrates. Although a great deal of information about cells and their responses to exogenous agents, such as therapeutic drugs, can be learned from these studies, there are also major limitations to this approach. In short, cells growing on plastic are flat, whereas cells in the body are very three-dimensional. Recent data from a number of laboratories demonstrate that cells growing in monolayers do not necessarily respond to exogenous substances in the same fashion as do cells growing in 3-D. Therefore, we are developing the technology for imaging human mammary epithelial cells growing in a three-dimensional reconstituted basement membrane. This technique will enable tracking fluorescently labeled proteins in living cells and will provide a way to evaluate "normal," premalignant and tumor cells. Using this approach we will be able to detect rapid, "real-time" responses by these cells to the effects of a spectrum of exogenous factors, including therapeutic agents.

(6) BODY

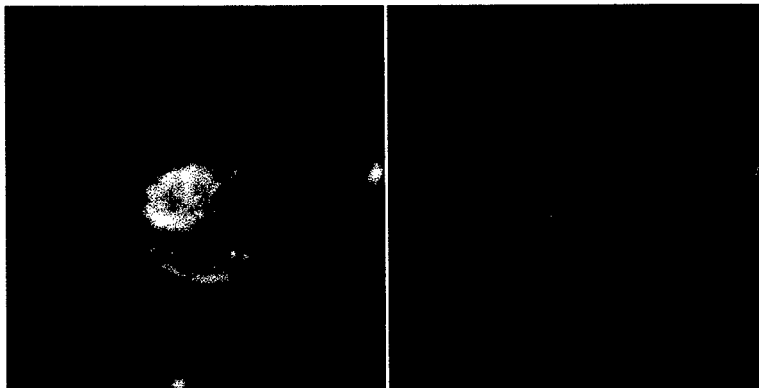
Specific Aim 1: Visualization Of Living Human Mammary Epithelial Cells Growing In A Three-Dimensional Matrix.

Human mammary epithelial cells growing in three-dimensional reconstituted basement membrane have not, to my knowledge, been examined in the living state using confocal microscopy. As a result, a significant amount of time was required to establish the parameters to be used for these studies. Our first step was to reduce the thickness of the reconstituted basement membrane used for the cultures in order to facilitate imaging while, at the same time, retaining appropriate cell behaviors. We had originally planned to do these measurements by examining fixed cells labeled with antibodies to known proteins. We decided that since fixatives can cause shrinkage that could distort the measurements, it was best to determine the appropriate thickness using live cells, since that was the way we ultimately wanted to image the cultures. These studies were done by incubating the cells in a dye known as DiOC₆(3) for several hours, then imaging them at various times over the next several days. Using this approach, we have determined that the cultures used for successful *in vivo* imaging of live cells need to be $\leq 50 \mu\text{m}$ thick.



Single optical section through a 3-D culture of normal human mammary epithelial cells growing in Matrigel. Cells were incubated in DiOC₆(3), a lipophilic dye that labels the cell surface and numerous cytoplasmic organelles.

During these studies we also realized that parameters such as laser intensity utilized for imaging, as well as the frequency of image collection, had a significant impact on the viability of these cell cultures. Multiple images were collected from these cultures to determine how rapidly the dye faded, an event referred to as photobleaching.



Optical section through cells labeled with DiOC₆(3).

Left: First image collected after 5 hrs incubation.

Right: Same cell after collecting 50 images, 1 every 5 minutes.

Significant photobleaching occurred after 50 images, as seen above, emphasizing the need to have a robust label with a strong signal. The next obvious question was what effect did this laser intensity have on cell viability? Cells experiencing this degree of laser exposure demonstrated problems with cell division and did not survive. Therefore, we are investigating other dyes that might have stronger signals that would require lower laser intensity.

Our ultimate goal is to track fluorescently labeled proteins of interest in the live cell cultures in order to follow the reorganization of proteins in response to externally applied agents. This requires the development of several different techniques. First, the fluorescently tagged proteins must be introduced into the cells. Several different approaches are being tested for transfecting the cells with the DNA, tagged with green fluorescent protein (GFP), encoding the protein of interest. Cells shown below have been transiently transfected with β -catenin-GFP by electroporation and were examined 48 hours later. The normal cells demonstrate β -catenin in the cytoplasm and the tumor cells demonstrate β -catenin in the nucleus (see below).



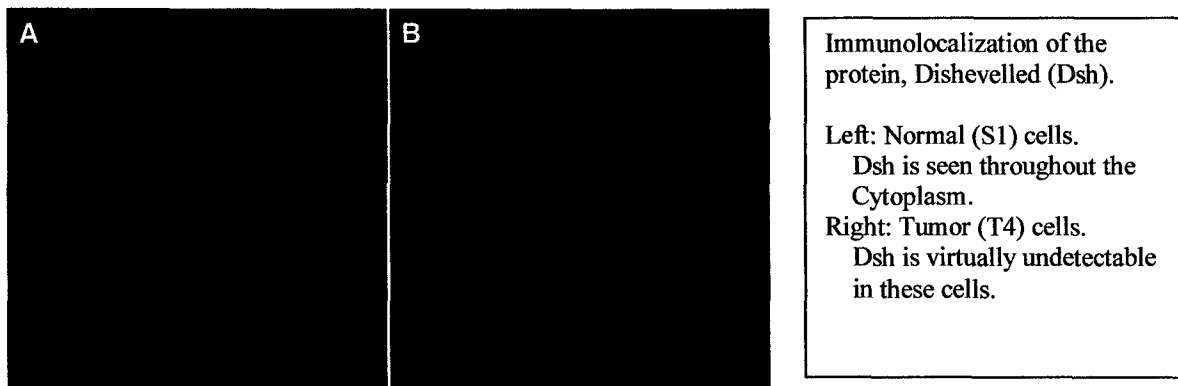
Living human mammary epithelial cells showing distribution of β -catenin-GFP.

Left, normal (S1) cells.

Right, tumor (T4) cells.

Unfortunately, these cells had very low survival rates following electroporation. Therefore, other means of transfecting cells, including virus and lipid vectors, are being tested.

Concurrent with the development of procedures for live-cell imaging, we are examining the distribution of proteins of the Wnt signaling pathway in fixed cells. The Wnt signaling pathway is an important pathway for normal development of vertebrate and invertebrate embryos. In response to a signal, one of the proteins in this pathway, β -catenin, travels to the nucleus and turns on gene transcription (Larabell et al., 1997; Molenaar et al., 1996). This same pathway has been implicated in certain cancers, where β -catenin has been found in the nucleus of tumor cells. In vitro studies show that the accumulation of β -catenin is regulated by an upstream protein known as Dishevelled (Dsh). We recently showed that Dishevelled travels along microtubules to one specific region of the embryo where it down-regulates GSK-3, a negative regulator of β -catenin, triggering the accumulation of cytoplasmic β -catenin (Miller et al., 1999). Therefore, we decided to examine the distribution of Dishevelled in the human mammary epithelial cells. We show that Dishevelled is distributed throughout the cytoplasm of the normal (S1) cells but is virtually undetectable in the tumor (T4) cells.



Our previous data showed that β -catenin is at cell-cell junctions in the normal (S1) cells whereas the tumor cells fail to form junctions and β -catenin is randomly distributed (Weaver et al., 1997). The lack of Dishevelled in the tumor cells is quite intriguing and will be investigated to determine if this is related to the lack of cell-cell junctions associated with tumorigenesis. Further experiments are required to explain these data, including Western blot analyses, to determine whether there is indeed a down-regulation of Dishevelled in these cells.

Specific Aim #2: Examination of the effects of exogenous factors on living human mammary epithelial cells growing in a three-dimensional matrix.

These studies have not yet begun. There are a number of procedures that must be developed and perfected in the live-cell imaging, as well as baseline studies that must be conducted, before we can begin testing the effects of exogenous factors on the living human mammary epithelial cells growing in Matrigel.

(7) KEY RESEARCH ACCOMPLISHMENTS:

- First report of imaging live human mammary epithelial cells grown in 3-D cultures.
- First imaging of β -catenin-GFP in 3-D cultures of live human mammary epithelial cells
- First report of a differential distribution of the protein Dishevelled, a protein in the Wnt signaling pathway capable of regulating β -catenin, in fixed cells.

(8) REPORTABLE OUTCOMES

There are no publishable outcomes at this time. We are in the early stages of developing techniques that are not yet appropriate for publication. In addition, data regarding the components of the Wnt signaling pathway are too preliminary and require additional research prior to publication.

(9) CONCLUSIONS

Live cell imaging of eggs and embryos has revealed data that could not readily be obtained using other techniques. We are developing similar techniques for imaging human mammary epithelial cells growing in 3-D cultures. We expect this approach will also yield valuable information about the responses of tumor cells to exogenous agents that would not otherwise be generated from studying fixed specimens. Studying cells growing in 3-D cultures in the living state is not trivial and has required numerous modifications. Obtaining information about the efficacy of those changes has been complicated by the fact that these cells grow very slowly and considerable time passes between making an adjustment and evaluating the consequences. Nonetheless, we have made considerable progress in this regard and have obtained preliminary data monitoring β -catenin-GFP in living breast cells, both normal and tumor. Once we understand the behavior of this protein in these cells growing in 3-D cultures, we can begin to evaluate its responses to the addition of components expected to modify its distribution in cells. These studies will lay the foundation for using this system to investigate potential therapeutic agents.

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(11) APPENDICES

NA

(12) BINDING

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